

Transformation of *Phomopsis viticola* with the Green Fluorescent Protein

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Abstract

Phomopsis viticola is the causal agent of Phomopsis cane and leaf spot on *Vitis* spp., which is a serious and economically important disease in temperate regions. Here we describe the transformation of this fungus with two different constructs (pBHt2_sGFP and pIGPAPA) containing the green fluorescent protein (GFP) and the hygromycin B resistance gene (*hph*). Protoplast mediated transformation yielded three fluorescent transformants (DA-2, DA-4, and DA-5) and multiple non-fluorescent transformants. All transformants obtained were mitotically stable. The growth rates of DA-4 and DA-5 were slightly, but significantly ($P < 0.05$), less than DA-2 and OH-22 (wild type); however, transformation resulted in no change in sporulation rate, log spore production, and virulence on grape internodes and leaves compared to the wild type. These transformants will be useful tools for elucidating fungal penetration of host plants, invasive growth, and the nature of its host association.

Introduction

Phomopsis viticola (Sacc.) Sacc. is the causal agent of Phomopsis cane and leaf spot on *Vitis* spp. This disease occurs worldwide, with the greatest economic losses experienced in moist temperate regions where it can reduce yields by as much as 30% (4, Ellis, *unpublished data*). *P. viticola* can produce necrotic lesions on leaves, internodes, and rachises, as well as rot berries (5). The histological detail of such infections is limited at best.

Reporter genes like the green fluorescent protein (GFP) allow for the visualization of fungal structures upon and within infected tissues. Here we used two GFP containing constructs, each with a different promoter, to transform *P. viticola*.

The successful GFP transformation of *P. viticola* reported here will provide the necessary tools for investigating host penetration, invasive growth, and reproduction by this important pathogen of grapes as well as defining the nature of the host-pathogen interaction.

Materials and Methods

Fungal strains and plasmids. *P. viticola* wild type strains OH-22, OH-9, MI-2, and Pv08001 were used in these studies. OH-22 was used for transformation, as it is fast growing and virulent. Transformation plasmids pBHt2_sGFP and pIGPAPA contain the GFP gene driven by the ToxA and ICL promoters respectively, as well as the hygromycin resistance gene (*hph*) driven by the *trpC* promoter.

Transformation. Protoplast-mediated transformation was adapted from De Guido et al (2). Protoplast transformation was performed on ice using *Hind*III digested plasmids and polyethylene glycol 3350 (PEG).

Protoplast recovery. Transformed protoplasts were recovered in SHV (0.6 M sucrose, 5mM HEPES 1mM (NH₄)₂HPO₄, 0.2 mM biotin, and 1 mM thiamine, pH 5.3) and selected for with hygromycin B. Eight days later, transformed colonies were isolated. **Figure 1** compares fluorescent transformants against the wild type (Nikon 80i).

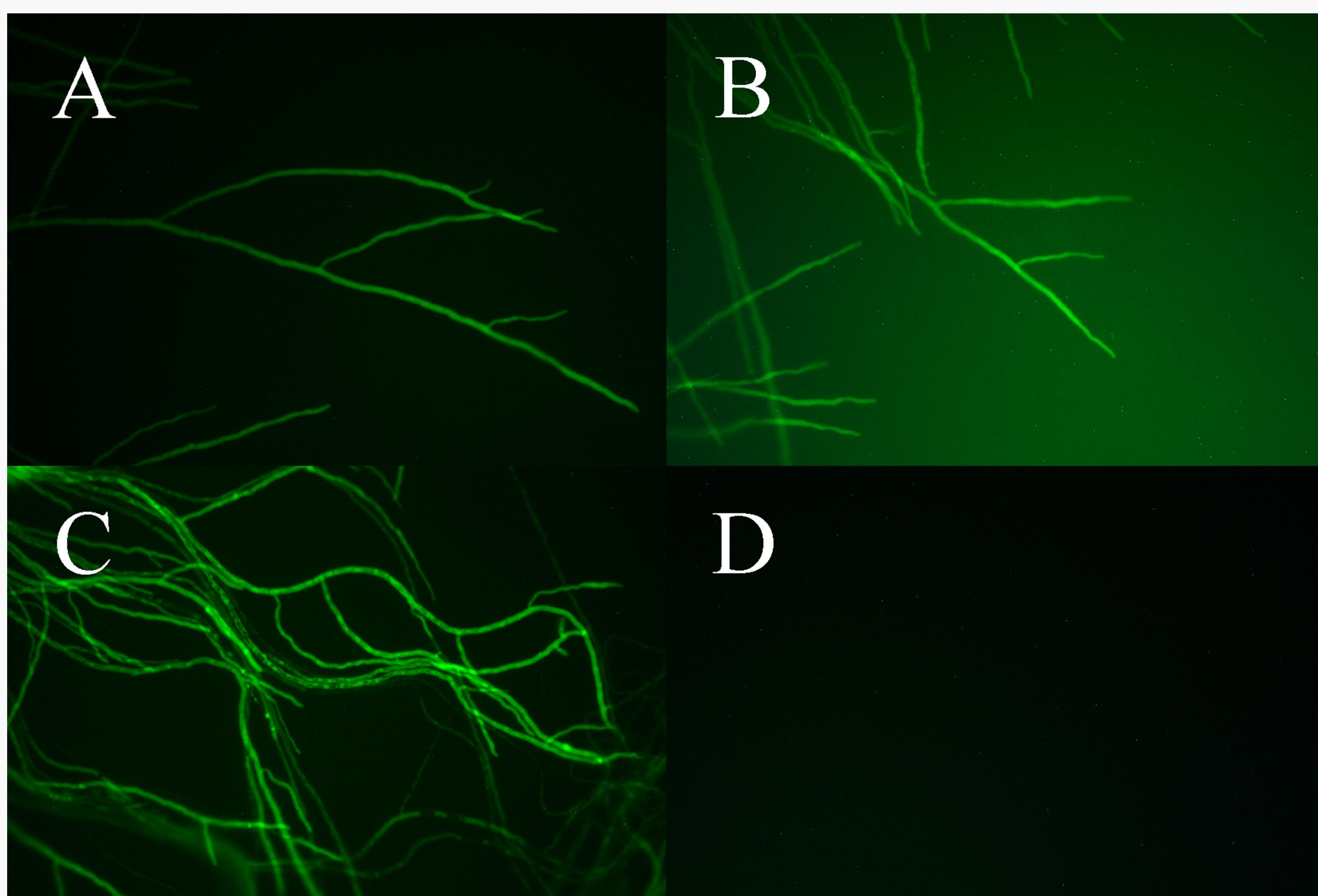


Figure 1. Epifluorescence micrographs of **A)** DA-2, **B)** DA-4, **C)** DA-5, and **D)** OH-22 (wild type).

Transformants vs. wild type. Growth rate, time to sporulate, and amount of spores produced after a fixed time were examined. Pathogenicity and virulence were examined on 'Seyval' grapes. Disease severity was measured 14 d after inoculation on canes (1) and on leaves (4) (**Figure 2**). Isolations were made from lesion margins on infected leaves and internodes.

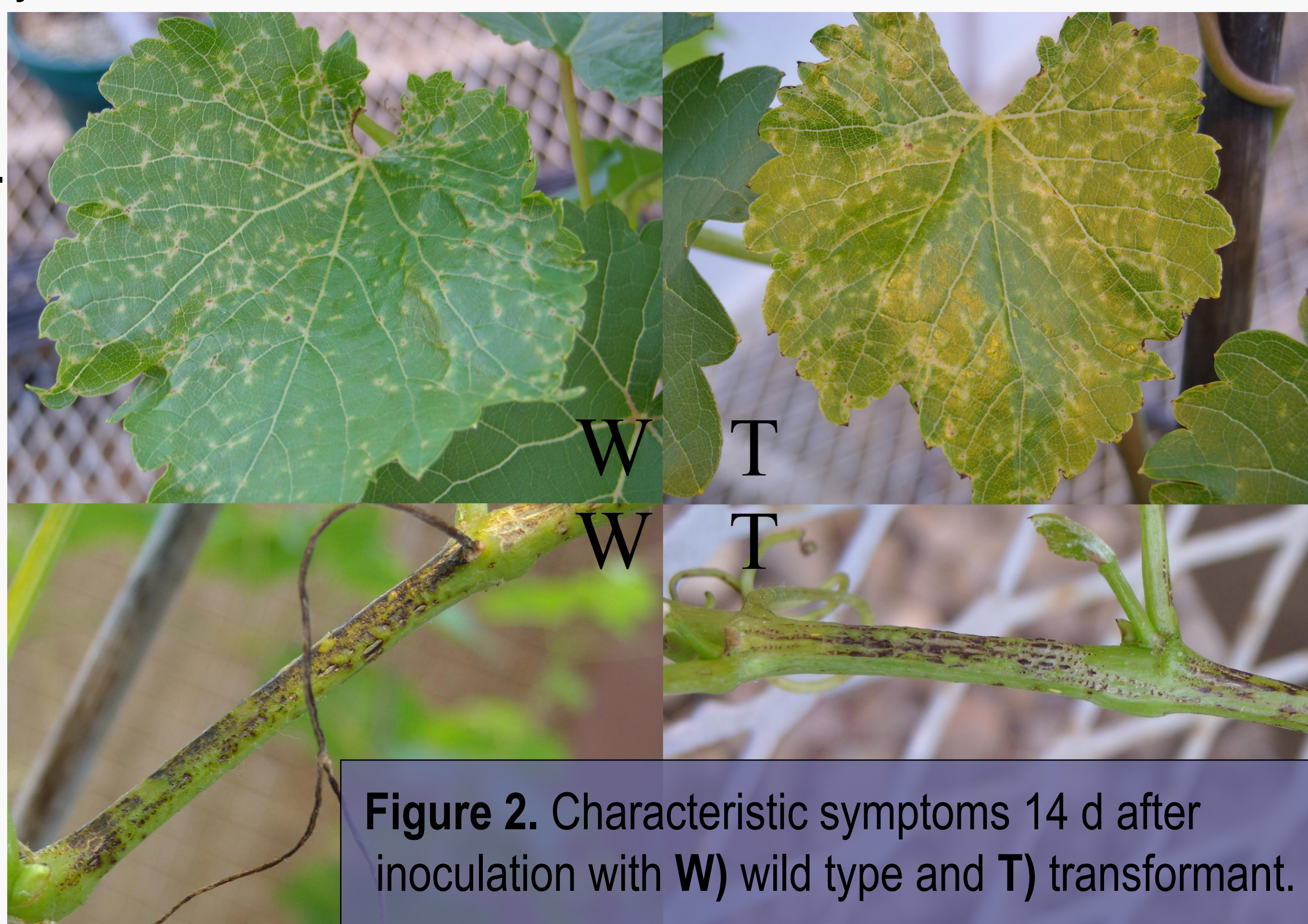


Figure 2. Characteristic symptoms 14 d after inoculation with **W)** wild type and **T)** transformant.

Visualization in grape.

‘Seyval’ grapes were inoculated with the transformants. Multiple tissues were examined after different times. **Figure 3** presents confocal laser scanning micrographs of these examinations (Leica TCS NT).

Results

Transformation. pBHt2_sGFP transformation yielded 4 hygromycin B resistant colonies (DA-1 through 4). Transformation with pIGPAPA yielded 3 hygromycin B resistant colonies (DA-5 through 8). Three transformants were GFP positive (DA-2, DA-4, and DA-5); **Figure 1** shows them compared to the wild type OH-22.

Growth and sporulation of transformants. Growth and sporulation of all strains did not statistically differ. The exception was the second repetition for growth: OH-22 and DA-2 grew significantly more than DA-4 and DA-5 ($P < 0.05$, Tukey’s pairwise). None of the strains produced β -conidia after 14 d of growth on potato dextrose agar (PDA).

Virulence of transformants. All strains produced characteristic Phomopsis cane and leaf spot symptoms simultaneously (**Figure 2**). There was no measurable difference in leaf lesions. Internode disease severity of the transformants was not significantly different from the wild type (Kruskal-Wallis test, $P \geq 0.15$). **To confirm Koch’s postulates, all four *P. viticola* strains used for virulence testing were re-isolated from infected tissues. Transformants were confirmed GFP positive.**

Visualization in grape. Fluorescent hyphae of transformants were observed on rachis tissue and within leaf, berry, and internodal tissues (**Figure 3**). Transformants were observed after short (1 – 3 d), intermediate (46 – 51 d), and long (>200 d) time periods after inoculation.

Conclusions and Discussion

1. The transformation efficiency (1 fluorescent transformant per 5 and 40 μg of pBHt2_sGFP and pIGPAPA, respectively) in this study was less than the lowest reported by De Guido et al (2) (5 – 10 transformants μg^{-1} of pAN7-1 DNA).

2. De Guido et al used REMI (restriction enzyme-mediated integration) to increase the number of transformants recovered. This technique has been shown to induce large genome rearrangements in some fungi. We ventured to use a more standard and potentially more stable transformation approach.

3. The growth of DA-4 and DA-5 was statistically less than DA-2 and OH-22 in one repetition; however, the growth, sporulation rate, log spore production, and virulence of all three transformants are statistically and phenotypically indistinguishable to OH-22.

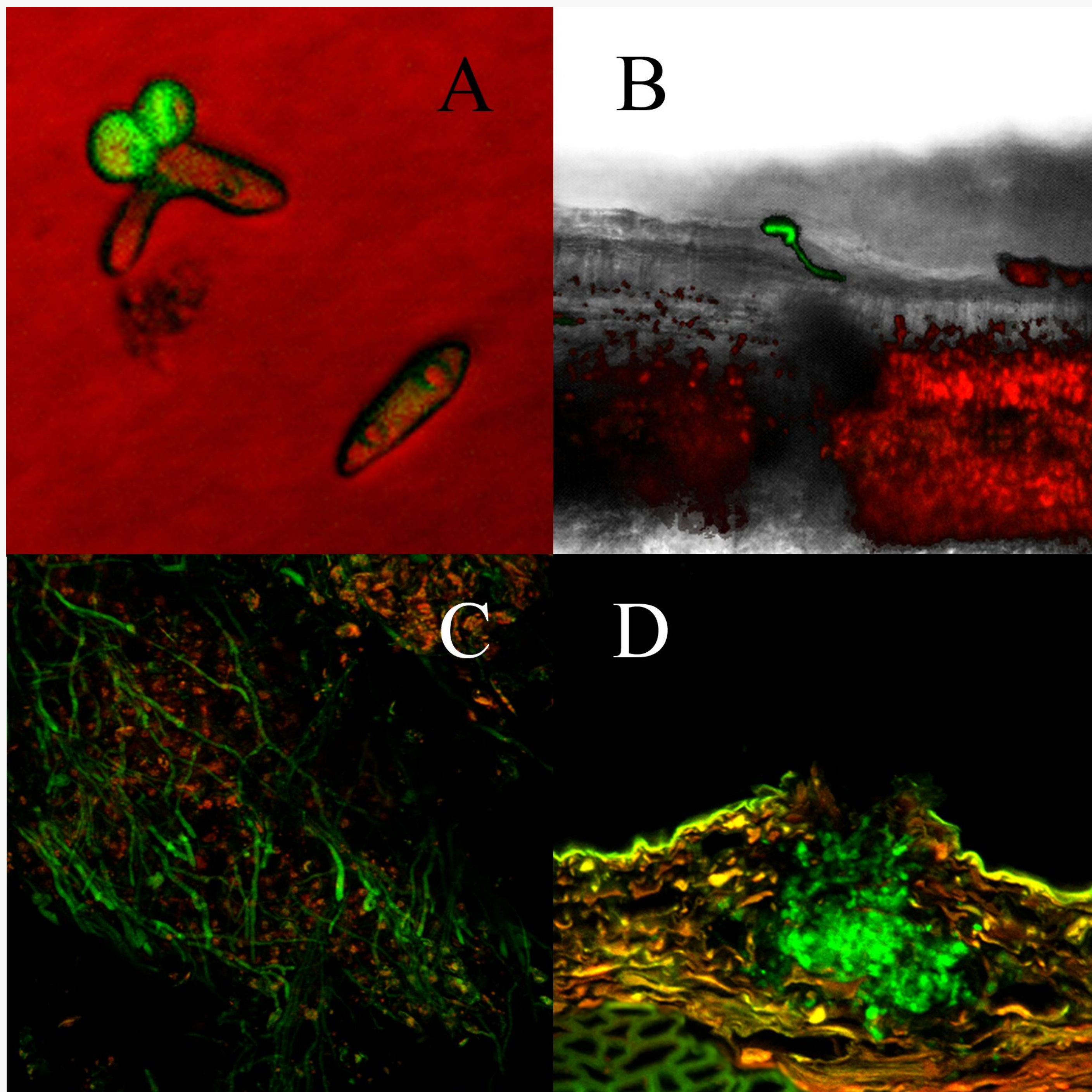


Figure 3. Confocal micrographs of transformants in grape. **A)** Surface of a rachis with fluorescent appressoria, 1 dpi. **B)** Leaf cross section; conidium appears to have elaborated an appressorium and penetrated the cuticle and upper epidermis; red structures are chloroplasts, 1 dpi. **C)** Berry cross section with intensive hyphal colonization beneath berry exocarp, 46 dpi. **D)** Cross section of grape internode showing a pycnidium with α -conidia of *P. viticola*; phloem and cuticle exhibit autofluorescence, 217 dpi.

4. We also demonstrated that the transformants retain their production of GFP during infection of grape. This, in combination with the retention of wild type characteristics, provides confidence these transformants will be useful tools in investigating host penetration, invasive growth, and reproduction by this important pathogen of grapes as well as defining the nature of the host-pathogen interaction.

Acknowledgements and References

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